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(54) Title: HUMAN TYPE II GONADOTROPIN-RELEASING HORMONE RECEPTOR

(57) Abstract

A human gonadotropin-releasing hormone receptor, and related compositions and methods are disclosed. The polypeptide has G protein-coupled receptor characteristics and, based on homology to other mammalian gonadotropin-releasing hormone receptors, appears to be the receptor for the conserved GnRH II ligand. The polypeptide may be used within methods to detect the natural human ligand and ligand analogs. The receptor can also be used within methods to influence sexual behavior and reduce proliferation of tumor cells.

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Description

HUMAN TYPE II GONADOTROPIN-RELEASING HORMONE RECEPTOR

Background of the Invention

Mammalian gonadotropin-releasing hormone (GnRH), a decapeptide, is secreted from the hypothalamus and controls the reproductive hormone cascade directing synthesis and release of pituitary gonadotropins through specific high-affinity membrane bound receptors. These gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), in turn regulate the activity of testes and ovaries. Because of its central role in the reproductive cascade, GnRH agonists and antagonists have been used for many therapeutic applications, including treatments for precocious puberty, endometriosis, uterine leiomyomata, hirsutism, infertility and as contraceptive agents.

In addition to regulating reproduction through the stimulation of pituitary gonadotropins, infusion of GnRH hereafter referred to as "Type I GnRH", or "GnRH-I", into discrete areas of the midbrain central grey directly affected reproductive behavior (sexual arousal) in ovariectomized estrogen-primed rats (Riskine and Moss, Research Bulletin 11:481-85, 1983; Dudley and Moss, Brain Research 411:161-67, 1988; and Kadar et al., Physiology & Behavior 51:601-05, 1992). This effect is independent of Type I GnRH effects on the pituitary, and implies that GnRH receptors exist in other brain areas outside the pituitary. This observation further suggests that Type I GnRH or a related molecule is produced in brain areas outside of the hypothalamus.

Type I GnRH was originally isolated from mammalian hypothalamus, and since then structural variants have been demonstrated in the brains of non-mammalian

vertebrates (for instance, catfish, dogfish, salmon, lamprey, and chicken). It is almost certain the Type I GnRH family ("GnRHs") will be characterized in the future.

GnRH proteins are highly conserved in length, and also at specific amino acid residues at the N terminus, (amino acid residues 1, 2, and 4) and at the C terminus, (amino acid residues 9 and 10). It has been shown that a gene duplication occurred early in evolution to produce hypothalamic variants of GnRH with a predominant function in regulating pituitary gonadotropins. A second form, Type II GnRH, has been highly conserved through vertebrate evolution, and is mainly extrahypothalamic (i.e., expressed in the midbrain region and maybe in the peripheral nervous system). This Type II GnRH (or GnRH II) was designated "chicken GnRH II (cGnRH II)", as it was first isolated from chicken (King and Millar, Cell. Mol. Neurobiol., 15:5-23, 1995). The exact function of cGnRH II is not known, however Chicken GnRH II was 100 times more potent than salmon GnRH, and 1000 times more potent than chicken GnRH I, mammalian GnRH, and lamprey GnRH in inhibiting the M-current (potassium current) in bullfrog sympathetic neurons. Thus, cGnRH II may act as a neuropeptide to mediate late, slow excitatory postsynaptic potential (Jones, Neurosci. Lett., 80:180-84, 1987). GnRH analogues have been observed to affect reproductive behavior in rats (Riskine and Moss, Research Bulletin 11:481-85, 1983; Dudley and Moss, Brain Research 411:161-67, 1988; and Kadar et al., Physiology & Behavior 51:601-05, 1992), and cGnRH II in ring doves (King and Millar, ibid) and in white-crowned sparrows when injected into the third ventricle. This suggests that cGnRH II may serve as a neurotransmitter or in a neuromodulatory role, perhaps to control reproductive behavior and may have applications in the central and sympathetic nervous systems.

The Type I GnRH receptor (Type I GnRH-R) has the structural characteristics of G protein-coupled

receptors, consisting of a single peptide chain containing seven hydrophobic transmembrane domains and connecting hydrophilic extracellular and intracellular loops. The coding region of the Type I GnRH-R is distributed over three exons, in contrast with many G protein-coupled receptors which are intronless. The human Type I GnRH-R has features that distinguish it from other G protein-coupled receptors: (a) the human Type I receptor lacks a cytoplasmic C-terminal tail; (b) it has a reciprocal interchange of amino acid residues N87 and D318; and (c) it has a change of the highly conserved DRY to DRS sequence at the intracellular juncture of the third transmembrane domain, creating a potential phosphorylation site. The mammalian Type I GnRH receptor has a high fidelity for the mammalian Type I GnRH and binds other GnRHs poorly, whereas non-mammalian vertebrate receptors are promiscuous in binding the various GnRH structural variants (Davidson et al., Mol. Cell. Endocrinol. 100:9-14, 1994; King and Millar, Cell. Mol. Neurobio. 15:5-23, 1995; and Sealfon and Millar, Cell. Mol. Neurobio., 15:25-42, 1995). Expression of the Type I GnRH-R has been reported to be predominantly in the hypothalamus, as well as in various human reproductive tissues (ovary and testes), non-reproductive tissues (placenta, breast, and pituitary), and human tumors and tumor cell lines (breast, prostate, endometrial, ovarian, kidney, and pancreatic tumors) (Kakar et al., Mol. Cell. Endocrinol., 106:145-49, 1994; Kakar and Jennes, Cancer Letts., 98:57-62, 1995).

GnRH binding sites have been demonstrated in various human carcinomas and tumor cell lines, including prosthetic and breast carcinomas and GnRH analogues exerted inhibitory effects on the growth of these cells (Eidne et al., J. Clin. Endocrinol. Metab., 64:425-32, 1987; Millar et al., SAMJ, 72:748-55, 1987; Harris et al., Progress in Cancer Research and Therapy, Vol. 35: Hormones and Cancer 3, Raven Press, Ltd., NY, 174-78, 1988; Harris et al., Canc. Res. 51: 2577-81, 1991; Limonta et al., J.

Clin. Endocrinol. Metab., 75: 207-12, 1992; Schally, Progress in Research and Practice: Fertility and Sterility, Parthenon Publishing Group, NY, 233-261, 1992; Emons and Schally, Hum. Repro. Update, 9:1364-79, 1994; and Conn, Annu. Rev. Med. 45:391-405, 1994). The affinity of the receptors for the classical GnRH ligand used for the pituitary receptor is usually low, suggesting that the receptor may be a variant of the Type I GnRH receptor.

GnRH has been shown to have direct effects on gonadal function (King and Millar, Cell. Molec. Neurobiol. 15:5-23, 1995), independent of stimulation of pituitary gonadotropins, and it is possible that a GnRH II receptor may also play a role in mediating these effects. Administration of cGnRH II has been shown to stimulate reproductive behavior. Given the high prevalence of sexual dysfunction and impotence in humans, a GnRH II receptor may find application in developing GnRH II analogs for the treatment of these conditions.

There is therefore a need in the art for a Type II gonadotropin-releasing hormone receptor which can serve to identify the corresponding ligand, and further define the distribution and role of this highly conserved receptor and its ligand. The receptors of the present invention can also serve to identify potential GnRH II agonists and antagonists which could be used as therapeutics in the treatment of sexual dysfunctions, and as potential chemotherapeutics in the treatment of various cancer types. The present invention fulfills this need by providing a human Type II gonadotropin-releasing hormone receptor (GnRH-R II), as well as related compositions and methods. The present invention provides these and other, related advantages.

Summary of the Invention

The present invention provides a novel human Type II gonadotropin-releasing hormone receptor polypeptide and related compositions and methods.

Within one aspect, the present invention provides an isolated polynucleotide encoding a mammalian type II gonadotropin-releasing hormone receptor polypeptide. Within one embodiment is an isolated polynucleotide which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:3. Within another embodiment is an isolated polynucleotide comprising the sequence of SEQ ID NO:2. Within a related embodiment is an isolated polynucleotide that is DNA.

Within a second aspect of the invention there is provided an expression vector comprising (a) a transcription promoter; (b) a DNA segment encoding a type II gonadotropin-releasing hormone receptor polypeptide, and (c) a transcription terminator, wherein the promoter, DNA segment, and terminator are operably linked. Within a related embodiment of the invention there is provided a cultured eukaryotic cell into which has been introduced an expression vector as disclosed above, wherein said cell expresses a protein polypeptide encoded by the DNA segment.

Within a third aspect of the invention is provided a method for producing a mammalian type II gonadotropin-releasing hormone receptor polypeptide comprising the steps of culturing a cell having an expression vector as discussed above which expresses a mammalian type II gonadotropin-releasing hormone receptor polypeptide and recovering the mammalian type II gonadotropin-releasing hormone receptor polypeptide.

Within a fourth aspect of the invention is provided an isolated polypeptide comprising a mammalian type II gonadotropin-releasing hormone receptor polypeptide. Within a related aspect is provided an antibody that specifically binds to a type II gonadotropin-releasing hormone receptor.

Within an additional aspect of the invention there is provided an antibody that specifically binds to a Zcytor4 polypeptide as disclosed above, and also an anti-

idiotypic antibody which neutralizes the antibody to a Zcytor4 polypeptide.

Within another aspect of the invention is provided a genomic DNA polynucleotide comprising the sequence of SEQ ID NO:1 which encodes a mammalian type II gonadotropin-releasing hormone receptor.

Within another aspect of the invention is provided a probe which comprises an oligonucleotide of at least 16 nucleotides which is at least 80% identical to the same length portion of SEQ ID NO:3 or a complement of a polynucleotide molecule that specifically hybridizes to SEQ ID NO:3.

Within a final aspect of the invention is provided a method for identifying a compound which modulates human type II gonadotropin-releasing hormone receptor-mediated metabolism in a cell, comprising the steps of incubating a test compound with eukaryotic cells which express recombinant type II human gonadotropin-releasing hormone receptor polypeptide on their surface; and measuring the metabolism of the cells in the presence and in the absence of the test compound, or measuring the effect of a test compound on receptor (+) and receptor (-) cells, wherein an increase in metabolism or effect above a control value indicates a test compound that modulates type II human gonadotropin-releasing hormone receptor mediated metabolism.

These and other aspects of the invention will become evident upon reference to the following detailed description and the attached drawing.

Brief Description of the Drawings

Figure 1 illustrates an alignment of the amino acid sequences of human GnRH-R I and human GnRH-R II. The putative boundaries of transmembrane domains 5 (TMV), 6 (TMVI) and 7 (TMVII) are indicated.

Figures 2 A, B, and C illustrate a multiple alignment of human arginine vasopression receptor 1

(HUMAVPRI), *Homo sapiens* oxytocin receptor (HSOXY), and human Type I gonadotropin-releasing hormone receptor (HUMGNRHR) and the partial sequence of human type II gonadotropin-releasing hormone receptor polypeptide (HUMGNRHRII). Consensus characters are ~ acidic, ! hydrophobic, @ amido, # aromatic, $ basic, % hydroxyl containing, ^ proline, and & sulfur containing. XXXXX denotes an intron 1 donor/acceptor site in the area of extracellular loop 1. The amino acid sequence KGSHAPAGEFAL denotes intron 2 donor/acceptor site in the area of intracellular loop 3. A coding sequence arising from one possible splice junction is:

KGSH GETPIPRP

SKF **APAGEFAL**

resulting in the junction sequence KGSHAPAGEFAL.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter:

Allelic variant : Any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (i.e., no change in the encoded polypeptide), or may encode polypeptides having altered amino acid sequence. The term "allelic variant" is also used herein to denote a protein encoded by an allelic variant of a gene.

Complements of polynucleotide molecules: Polynucleotide molecules having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

Expression vector: A DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that

provide for its transcription. Such additional segments may include promoter and terminator sequences, and optionally one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

Isolated: When applied to a protein, the term "isolated" indicates that the protein is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated protein is substantially free of other proteins, particularly other proteins of animal origin. It is preferred to provide the proteins in a highly purified form, i.e., greater than 95% pure, more preferably greater than 99% pure. When applied to a polynucleotide molecule, the term "isolated" indicates that the molecule is removed from its natural genetic milieu, and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, and may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316:774-78, 1985).

Operably linked: As applied to nucleotide segments, the term "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

Receptor: A cell-associated protein, or a polypeptide subunit of such protein, that binds to a bioactive molecule (the "ligand") and mediates the effect of the ligand on the cell. Binding of ligand to receptor results in a change in the receptor (and, in some cases, receptor multimerization, i.e., association of identical or different receptor subunits) that causes interactions between the effector domain(s) of the receptor and other molecule(s) in the cell. These interactions in turn lead to alterations in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, cell proliferation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. The GnRH-R II has characteristics of G protein-coupled receptors, as discussed in more detail below.

The present invention is based in part upon the discovery of a novel DNA sequence that encodes a polypeptide having homology to the human Type I gonadotropin-releasing hormone receptor. An isolated human genomic DNA fragment encoding two putative exons, corresponding to exon 2 and exon 3 of the Type I GnRH-R, and a putative intron of this receptor is shown in SEQ. ID. NO:1. The deduced amino acid sequence indicates that the encoded receptor polypeptide possesses G protein-coupled receptor characteristics, and has been designated human Type II gonadotropin-releasing hormone receptor, human GnRH-R II, or GnRH II receptor. This receptor is suspected to be the receptor for the human equivalent of chicken GnRH II.

The human Type I GnRH receptor is a G protein coupled receptor consisting of a single peptide chain containing seven transmembrane domains connected by hydrophilic extracellular and intracellular loops. The coding region for the Type I GnRH receptor, in contrast to

many other G protein coupled receptor genes, is spread over three exons. The genomic organization of the mouse and human GnRH receptor shows intron 1 to be located near the 3' end of the fourth transmembrane domain and the second intron in intracellular loop 3. Exon 1 encodes approximately half of the gene with exon 2 and 3 encoding the remainder. The sequences of six mammalian Type I GnRH receptors have been reported, human, ovine, bovine, porcine, rat and murine. The amino acid sequences of these receptors are more than 85% conserved overall, and are nearly identical within the transmembrane domains (Sealfon and Millar, Cell. Mol. Neurobiology 15: 25-42, 1995). The human Type I receptor has been localized to chromosome 4 (4q13.1-q21.1), and the mouse receptor to chromosome 5 (Kaiser et al., Genomics 20:506-08, 1994).

Based on comparison to mammalian Type I GnRH receptors, the GnRH-R II polypeptide shown in SEQ. ID. NO:3 comprises transmembrane domains 5, 6, and 7; a second and third extracellular domain and a third intracellular domain; and a C-terminal sequence containing a stop codon. These transmembrane domains have between 60 to 70% homology to the human Type I GnRH receptor. The intracellular domains show more variability when compared to the human GnRH I receptor, which is characteristic of G protein-coupled receptors, since these intracellular regions interact with various downstream proteins and not with the receptor ligands (Figure 1). Those skilled in the art will recognize that these domain boundaries are approximate, and are based on alignments with known proteins and predictions of protein folding.

An intron of about 400 bp appears in the third intracellular loop. The 3' end of the first intron appears just before the second extracellular domain, suggesting it is within or adjacent to the fourth transmembrane domain, as would be predicted based upon the human Type I GnRH receptor sequence. Two putative acceptor sites are present (Figures 2A, 2B and 2C) In

addition, the figures also show consensus amino acid residues between human GnRH-R II and three amino acid sequences predicted to be similar to human GnRH-R II. Human GnRH-R II has been localized to chromosome 1 (1q12-21).

Based on the Type I GnRH receptor, the human GnRH-II receptor may be tissue-specific. The genomic DNA sequence contains an intron, and therefore is in an unprocessed form *in vivo* until activated, most likely in a tissue-specific manner. In the case of most brain-specific receptors, ligand and receptor are present in the tissue in low concentrations, and little if any ligand can be detected in the circulation. The specific localization of both human Type I and type II GnRH receptors and ligands suggests that anatomy may convey specificity. That is the ligand for a corresponding receptor may be specifically produced only in the vicinity of the receptor, and the receptor may activate only when ligand is present.

Within preferred embodiments of the invention the isolated polynucleotides will hybridize to similar sized regions of SEQ ID NO:2, or a sequence complementary thereto, under stringent conditions. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is at least about 0.02 M at pH 7 and the temperature is at least about 60°C.

Methods for isolating DNA and RNA are well known in the art. Within the preferred invention, it is generally preferred to isolate RNA from brain or testis, although DNA can also be prepared using RNA from other tissues or isolated as genomic DNA. Total RNA can be prepared using guanidine HCl extraction, followed by

isolation by centrifugation in a CsCl gradient (Chirgwin et al., Biochemistry 18:52-94, 1979). Poly (A)+ RNA is prepared from total RNA using the method of Aviv and Leder (Proc. Natl. Acad. Sci. USA 69:1408-12, 1972). Complementary DNA (cDNA) is prepared from poly(A)+ RNA using known methods. Polynucleotides encoding GnRH-R II polypeptides are then identified and isolated by, for example, hybridization or PCR.

The sequence of a polynucleotide molecule encoding a representative human GnRH-R II polypeptide is shown in SEQ ID NO: 2, and the corresponding amino acid sequence is shown in SEQ ID NO: 3. Those skilled in the art will recognize that these sequences correspond to one allele of the human gene, and that allelic variation is expected to exist. Allelic variants can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the DNA sequence shown in SEQ ID NO: 2, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NO: 3.

The present invention further provides counterpart receptors and polynucleotides from other species ("species orthologs"). Of particular interest are GnRH type II receptors from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate receptors. Species orthologs of the human GnRH II receptor can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses the receptor. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A receptor-encoding cDNA

can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequence. A cDNA can also be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent No. 4,683,202), using primers designed from the sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to the receptor. Similar techniques can also be applied to the isolation of genomic clones.

The present invention also provides isolated receptor polypeptides that are substantially homologous to the receptor polypeptide of SEQ ID NO: 3 and its species orthologs. The term "substantially homologous" is used herein to denote polypeptides having 50%, preferably 60%, more preferably at least 80%, sequence identity to the sequence shown in SEQ ID NO:3 or its species orthologs. Such polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO:3 or its species orthologs. Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-16, 1986; and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-19, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown in Table 1 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{\left[\begin{array}{l}\text{length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences}\end{array}\right]} \times 100$$

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Table 1

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Substantially homologous proteins and polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 2) and other substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification (an affinity tag), such as a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991), glutathione S transferase (Smith and Johnson, Gene 67:31, 1988), or other antigenic epitope or binding domain. See, in general, Ford et al., Protein Expression and Purification 2: 95-107, 1991, which is incorporated herein by reference. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

Table 2

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine

Table 3, continued

Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

Essential amino acids in the receptor polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244, 1081-85, 1989; Bass et al., Proc. Natl. Acad. Sci. USA 88:4498-502, 1991). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g., ligand binding and signal transduction) to identify amino acid residues that are critical to the activity of the molecule. Sites of ligand-receptor interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity; in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., Science 255:306-12, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992. The identities of essential amino acids can also be inferred from analysis of homologies with related receptors.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-57, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-56, 1989). Briefly, these

authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-37, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

Mutagenesis methods as disclosed above can be combined with high-throughput screening methods to detect activity of cloned, mutagenized receptors in host cells. Mutagenized DNA molecules that encode active receptors or portions thereof (e.g., ligand-binding fragments) can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

The receptor polypeptides of the present invention, including full-length receptors polypeptides, receptor fragments (e.g., ligand-binding fragments), and fusion polypeptides, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; and Ausubel et al., ibid., which are incorporated herein by reference.

In general, a DNA sequence encoding a GnRH-R II polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a GnRH-R II polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of the receptor, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is joined to the GnRH-R II DNA sequence in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Cultured mammalian cells are preferred hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-45, 1982), DEAE-dextran mediated transfection (Ausubel et al.,

eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987), and liposome-mediated transfection (Hawley-Nelson et al., Focus 15:73, 1993; Ciccarone et al., Focus 15:80, 1993), which are incorporated herein by reference. The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134, which are incorporated herein by reference. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) and Chinese hamster ovary (e.g., CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978, which are incorporated herein by reference) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as

"amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g., hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used.

Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222; Bang et al., U.S. Patent No. 4,775,624; and WIPO publication WO 94/06463, which are incorporated herein by reference. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., J. Biosci. (Bangalore) 11:47-58, 1987.

Fungal cells, including yeast cells, and particularly cells of the genus *Saccharomyces*, can also be used within the present invention, such as for producing receptor fragments or polypeptide fusions. Methods for transforming yeast cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075, which are incorporated herein by reference. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in yeast is the POT1 vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be

selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092, which are incorporated herein by reference) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454, which are incorporated herein by reference. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., J. Gen. Microbiol. 132:3459-65, 1986; and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349, which is incorporated herein by reference. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228, which is incorporated herein by reference. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533, which is incorporated herein by reference.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the

selectable marker carried on the expression vector or co-transfected into the host cell.

Novel receptors can be produced by a cultured cell, and the cell is used to screen for ligands for the receptor, including the natural ligand, as well as agonists and antagonists of the natural ligand. To summarize this approach, a cDNA or gene encoding the receptor is combined with other genetic elements required for its expression (e.g., a transcription promoter), and the resulting expression vector is inserted into a host cell. Cells that express the DNA and produce functional receptors are selected and used within a variety of screening systems.

Cells expressing functional receptor are used within screening assays. A variety of suitable assays are known in the art. These assays are based on the detection of a biological response in a target cell. An increase in metabolism above a control value indicates a test compound that modulates human gonadotropin releasing hormone type II receptor mediated metabolism. One such assay is a cell proliferation assay. Cells are cultured in the presence or absence of a test compound, and cell proliferation is detected by, for example, measuring incorporation of tritiated thymidine or by colorimetric assay based on the metabolic breakdown of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mosman, J. Immunol. Meth. 65: 55-63, 1983). An additional assay method involves measuring the effect of a test compound on receptor (+), containing the receptor of interest on their cell surface, and receptor (-) cells, those which do not express the receptor of interest. These cells can be engineered to express a reporter gene. The reporter gene is linked to a promoter element that is responsive to the receptor-linked pathway, and the assay detects activation of transcription of the reporter gene. A preferred promoter element in this regard is a serum response element, or SRE (see, e.g., Shaw et al., Cell 56:563-72,

1989). A preferred such reporter gene is a luciferase gene (de Wet et al., Mol. Cell. Biol. 7:725, 1987). Expression of the luciferase gene is detected by luminescence using methods known in the art (e.g., Baumgartner et al., J. Biol. Chem. 269:29094-29101, 1994; Schenborn and Goiffin, Promega Notes 41:11, 1993). Luciferase activity assay kits are commercially available from, for example, Promega Corp., Madison, WI. Target cell lines of this type can be used to screen libraries of chemicals, cell-conditioned culture media, fungal broths, soil samples, water samples, and the like. For example, a bank of cell-conditioned media samples can be assayed on a target cell to identify cells that produce ligand. Positive cells are then used to produce a cDNA library in a mammalian expression vector, which is divided into pools, transfected into host cells, and expressed. Media samples from the transfected cells are then assayed, with subsequent division of pools, re-transfection, subculturing, and re-assay of positive cells to isolate a cloned cDNA encoding the ligand.

A natural ligand for the GnRH-R II could be cloned from tissue where the Type II GnRH receptor has been found. One method would involve using PCR to identify ligands from tissue where the receptor is found, such as midbrain. Immunological methods could also be used. Once the ligand has been identified, synthetic analogs could be made. The pharmacology of the synthetic peptides can be tested in cells expressing the Type II GnRH receptor. It is likely that the natural ligand is cGnRH II since this peptide is conserved throughout evolution and has been described in mammals. However, it is possible that the human ligand may have minor variations from the cGnRH II structure.

Cells found to express the ligand are then used to prepare a cDNA library from which the ligand-encoding cDNA can be isolated as disclosed above. The present invention thus provides, in addition to novel receptor

polypeptides, methods for cloning polypeptide ligands for the receptors.

The GnRH Type II receptor is predicted to be the target of the Type II GnRH ligand (GnRH-II). cGnRH-II has been implicated as a neuromodulator and a possible chemotherapeutic and agonists (including the natural ligand) and antagonists to the human GnRH-II receptor would have an enormous potential in both *in vitro* and *in vivo* applications. Based on its role in the reproductive hormone cascade, many thousands of Type I GnRH analogs have been synthesized, and some of these are employed as therapeutic agents for the treatment of a diverse group of hormone-dependent diseases. Compounds identified as human GnRH-II receptor agonists and antagonists would be useful as therapeutic agents for hormone-dependent cancers. For example, agonist compounds could be used to inhibit cellular response to GnRH stimulation by desensitizing the receptor to the ligand. Agonists are thus useful in specifically inhibiting growth and/or development of mammary tumor cells bearing GnRH-R II in culture, or *in vivo*. Antagonists can be used to out-compete endogenous GnRH-II. Agonists and antagonists may also prove useful in the study of GnRH-II-directed neuromodulation, in particular, the effect of GnRH-II-directed neuromodulation of sexual behavior. Antagonists are also useful as research reagents for characterizing sites of ligand-receptor interaction. GnRH-II receptor agonists may find application in the treatment of diminished libido and impotence.

GnRH-R II polypeptides may also be used within diagnostic systems for detection of the receptor or for ligand localization and concentration in various tissues. Expression levels of the receptor may change in potentially tumorous tissue and detection could be used as a diagnostic.

Ligand-binding receptor polypeptide can be used for purification of ligand. The receptor polypeptide is

immobilized on a solid support, such as beads of agarose, cross-linked agarose, glass, cellulosic resins, silica-based resins, polystyrene, cross-linked polyacrylamide, or like materials that are stable under the conditions of use. Methods for linking polypeptides to solid supports are known in the art, and include amine chemistry, cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, and hydrazide activation. The resulting media will generally be configured in the form of a column, and fluids containing ligand are passed through the column one or more times to allow ligand to bind to the receptor polypeptide. The ligand is then eluted using changes in salt concentration, chaotropic agents ($MnCl_2$), or pH to disrupt ligand-receptor binding.

GnRH-R II polypeptides can also be used to prepare antibodies that specifically bind to GnRH-R II polypeptides. As used herein, the term "antibodies" includes polyclonal antibodies, monoclonal antibodies, antigen-binding fragments thereof, proteolytic or recombinant and the like, including genetically engineered antibodies. Antibodies are defined to be specifically binding if they bind to a GnRH-R II polypeptide with a K_a of greater than or equal to $10^7/M$. The affinity of a monoclonal antibody can be readily determined by one of ordinary skill in the art (see, for example, Scatchard, Ann. NY Acad. Sci. 51: 660-72, 1949).

Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; and Hurrell, J. G. R., Ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL, 1982, which are incorporated herein by reference). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals, such as horses, cows, goats, sheep, dogs,

chickens, rabbits, mice, and rats. The immunogenicity of a GnRH-R II polypeptide may be increased through the use of an adjuvant, such as Freund's complete or incomplete adjuvant. A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to GnRH-R II polypeptides. Exemplary assays are described in detail in Antibodies: A Laboratory Manual, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radio-immunoassays, radio-immunoprecipitations, enzyme-linked immunosorbent assays (ELISA), dot blot assays, inhibition or competition assays, and sandwich assays.

Antibodies to GnRH-R II are may be used for tagging cells that express the receptor, for affinity purification, within diagnostic assays for determining circulating levels of soluble receptor polypeptides, and as antagonists to block ligand binding and signal transduction *in vitro* and *in vivo*.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1

Identification of the Human Type II Gonadotropin Releasing Hormone Receptor Polypeptide

Oligonucleotide primers ZG 10,063 (SEQ ID NO: 4) and ZG 10,071 (SEQ ID NO: 5) were designed from the sequences of two expressed sequence tags (ESTs) in a DNA database. Analysis of the EST sequences suggested that they represented a portion of the extracellular loop 3, the putative stop codon, and a 3' untranslated region of a human type II GnRH receptor. The primers were used to obtain a 415 bp fragment that spans the region from the putative sixth transmembrane domain through the C-terminus

of the protein. A panel of "marathon ready" cDNA templates was prepared using a Marathon~ cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the protocol provided by the manufacturer, and human lymph node, placenta, uterus, liver, kidney, spleen and fetal brain DNA. These templates were used in polymerase chain reactions to generate DNA encoding human GnRH-II receptor.

PCR amplification was carried out according to manufacturer's instructions. Thirty pmol of each primer was used in the reactions. Each PCR template was amplified for 35 cycles (95°C, 20 seconds; 68°C, 1 minute) followed by a 10 minute extension at 72°C. A 415 bp fragment was found in all cDNAs tested, with the strongest signal found in placenta, uterus and liver. Sequence analysis of the 415 bp fragment provided a total of 361 nucleotides of readable sequence, which overlapped and confirmed the EST sequences and contained the stop codon.

The 415 bp fragment and primers ZG 10,063 (SEQ ID NO: 4) and ZG 10,071 (SEQ ID NO: 5) were used to screen a P1 genomic library. Three P1 clones, average size 80-100 kb, were identified: DMPC-HFF#1-0375-A7 (9792), DMPC-HFF#1-0549-F3 (9793) and DMPC-HFF#1-0940-H12 (9794).

Placenta, uterus, and liver "marathon ready" cDNA library templates were used for 5' RACE (rapid amplification of cDNA ends) to obtain upstream sequence information. Twenty pmol of primer ZC 10,070 (SEQ ID NO: 6) and AP1, supplied with the 5' RACE amplification kit (Clontech), were used and the 5' RACE was carried out according to the manufacturer's instructions. The reactions were initially incubated at 94°C for 1 minute, followed by 35 cycles (94°C, 20 seconds; 68°C, 5 minutes) followed by a 10 minute extension at 72°C. The 5' RACE reaction resulted in a band of about 1.4 kb in all tissues.

Sequence analysis of the 1.4 kb 5' RACE fragment and further EST sequences revealed an approximately 430 bp stretch of intronic sequence between the putative fifth

transmembrane domain (TM5) and the intracellular loop 3. Based on comparison with human type I GnRH receptor sequence, this intron is predicted to be intron 2, and has been found in all tissues tested and in all ESTs spanning this region. Sequence analysis of the 1.4 kb fragment further reveled the entire TM5, extracellular loop 2, and a portion of intron 1. Together with the 415 bp fragment, the genomic DNA coding sequence spanning extracellular loop 2 through the C-terminus of human GnRH-R II has been identified (SEQ. ID. NO. 1).

Example 2
Tissue Distribution

Northern analysis was performed using human brain-specific Northern blots and human fetal tissue-specific Northern blots from Clontech. The 415 bp DNA fragment was electrophoresed on a 1% agarose gel, the fragment was electroeluted, and then radioactively labeled using a random priming MEGAPRIME DNA labeling system (Amersham, Arlington Heights, IL) according the manufacturer's specifications. The probe was purified using a NUCTRAP push column (Stratagene Cloning Systems, La Jolla, CA). EXPRESSHYB (Clontech) solution was used for prehybridization and as a hybridizing solution for the Northern blots. Hybridization took place overnight at 68°C, and the blots were then washed in 2X SSC and 0.05% SDS at RT, followed by a wash in 0.1X SSC and 0.1% SDS at 50°C. Three transcript sizes were detected. Two transcript sizes were observed in all areas of the brain tested, one at approximately 3 kb and one at 6 kb. Signal intensity was highest for the 6 kb transcript. A third transcript size of 7.5 kb was present at varying intensity, with highest expression in cerebral cortex, medulla, spinal cord and corpus callosum. On the fetal blot, the highest level of expression of all three transcripts was in fetal kidney.

The same probe was used to probe human multiple tissue Northern blots (MTN, MTN II and MTN III, Clontech) using the same hybridization and washing conditions described above. Three transcript sizes, 3, 6 and 7.5 kb, were seen in varying intensity in all tissues, with the highest expression of the 3 and 6 kb transcripts in skeletal muscle, ovary, thyroid and spinal cord. The 7.5 transcript was most intense in spinal cord, brain, ovary, skeletal muscle and testis. A more stringent wash at 65°C did not alter the binding patterns described above.

Example 3
PCR-Based Chromosomal Mapping of the GnRH-R II Gene

The human GnRH-R II gene was mapped to human chromosome 1 by PCR using the Human/Rodent Somatic Cell Hybrid Mapping Panel Number 2 (National Institute of General Medical Sciences, Cornell Institute of Medical Research, Camden, NJ). The panel consisted of DNA isolated from 24 human/rodent somatic cell hybrids each retaining one specific human chromosome and the parental DNAs. Specific GnRH Type II receptor gene oligonucleotide primers, sense ZC 10,063 (SEQ. ID. NO. 4), and antisense ZC 10,071 (SEQ. ID. NO. 5), were used for PCR amplification. A 50 µl PCR reaction mixture was then prepared containing 10 µl DNA template, 5 µl 10X KlenTaq PCR reaction buffer (Clontech), 4 µl dNTPs mix (2.5 mM each; Perkin-Elmer Cetus, Norwalk, CT.), 50 pmol each ZC 10,063 (SEQ. ID. NO. 4) and ZC 10,071 (SEQ. ID. NO. 5), and 1 µl 50X Advantage KlenTaq Polymerase Mix (Clontech).

Fluorescence *In Situ* Hybridization and Subchromosomal Mapping of the Human GnRH-R II Gene

The GnRH-R II gene was mapped to the 1q12-21 region of chromosome 1 using fluorescence *in situ* hybridization as follows. A GnRH-R II specific probe was prepared using PCR. To a final volume of 50 µl was added 1 µg P1 DNA #9792, 5 µl 10X nick translation buffer (0.5 M

Tris/HCl, 50 mM $MgCl_2$, and 0.5 mg/ml BSA (nuclease free)), 5 µl dNTPs solution (0.5 mM dATP, 0.5 mM dGTP, and 0.5 mM dCTP), 5 µl 5 mM Bio-11-dUTP, 5 µl 100 mM DTT, 5 µl DNase I (1000X dilution of a 10 U/µl RNase-free stock: Boehringer Mannheim, Indianapolis, IN), and 12.5 U DNA polymerase I. The mix then was and incubated at 15°C for 1 hour in a Boekel microcooler (Feasterville, PA). The reaction was terminated by addition of 5 µl 0.5 M EDTA, pH 7.4. The probe was purified using G-50 DNA purification spin columns (Worthington Biochemical Co., Freehold, NJ) according the manufacturer's instructions.

Slide Preparation

Metaphase chromosomes were obtained from HEL cell culture. Cells were cultured in 100 x 15 mm culture dishes at 37°C, 5% CO_2. To prepare cells for harvest, 100 µl colemid (10 µl/ml stock; GIBCO BRL, Gaithersburg, MD.) was added to the culture medium and incubated at 37 °C for 2.5 to 3 hours. The medium was then removed and the cells were rinsed with 2 ml 1X PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2). Cells were removed from the plate with 2 ml trypsin (GIBCO), and centrifuged at 1000 rpm for 8 minutes, (Beckman, Palo Alto, CA; Model TJ-6 centrifuge, TH-4 swinging-bucket rotor). The supernatant was removed and the cells resuspended in 8 ml 0.075 M KCl (prewarmed to 37°C) and incubated in a 37°C waterbath for 10 minutes. The cells were pelleted by centrifugation (Beckman TJ-J, TH-4 swing bucket rotor), at 1,100 rpm for 5 minutes and resuspended in 8 ml of cold methanol:acetic acid (3:1), added dropwise with mixing, to fix the cells. The cells were incubated at 4°C for 20 minutes followed by centrifugation, (Beckman TJ-J, TH-4 swing bucket rotor), at 1,100 rmp for 5 minutes. The fixation process was repeated two more times without the 4°C incubation.

Frosted glass slides (VWR, Seattle, WA) were precleaned, and 5 µl 50% acetic acid was spotted on each

slide, followed by 5 µl of the fixed cell suspension. The slides were allowed to air dry at room temperature followed by incubation in a 42 °C oven overnight (Boekel). Cells were scored for suitable metaphase spreads using a microscope equipped with a phase contrast condenser.

Some metaphase chromosome preparations were ASG (acetic/saline/giemsa) G-banded (Sumner, et al., Nature New Biol., 232: 31-32, 1971) with Gurr's improved R66 Giemsa's Stain (BDH Laboratory Supplies, Poole, England). Suitable G-band chromosomes were photographed prior to hybridization experiments as follows: Slides containing suitable chromosome preparations can be used at room temperature, or following a 45-60 minute incubation at 90°C. Slides are then incubated for 2 hours in 2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), followed by a rinse in dH_2O, and stained in 5% Gurr's Giemsa stain diluted in 10% Gurr's Giemsa buffer solution, pH 6.8 (BDH Laboratory Supplies), prefiltered through Whatman #1 paper. G-banded metaphase chromosome spreads are visualized using an Olympus BH2-RFC microscope (Lake Success, NY). To use the same metaphase chromosome spreads for hybridization experiments, the chromosomes were destained in 100% EtOH for 20 minutes and air dried.

Hybridization of Metaphase Chromosomes

To a 1.5 ml Eppendorf tube was added 5 µg human cot-1 DNA (GIBCO), 200 ng biotin labeled P1 DNA 9792, 100 µg salmon testes DNA (Sigma Chemical Co., St. Louis, MO.), 0.1 volume 3M NaOAc and 2 volumes ethanol. This hybridization mix was vacuum dried in a Savant DNA SpeedVac DNA110 (Hicksville, NY). The dried pellet was resuspended in 10 µl hybridization solution (10% dextran sulfate, 2X SSC, and 50% formamide (EM Science, Houston, TX). The hybridization mix was denatured at 70-80°C for 5 minutes, followed by cooling on ice and pre-annealing at 37°C for 1-2 hours. Chromosome spreads were denatured by immersing each slide in denaturing buffer (70% formamide,

2X SSC) at 70-80°C for 5-10 minutes. The slides were then air dried at room temperature and prewarmed to 42°C just prior to addition of 20 µl of hybridization solution. The chromosomes were then covered with a coverslip and incubated at 37°C overnight in a moist chamber. The slides were then washed 3 times in 2X SSC containing 50% formamide at 42°C for 5 minutes, followed by 3 washes in 2X SSC at 42 °C for 5 minutes, then one wash in 4X SSC containing 0.05% Tween-20 (Sigma), for 3 minutes at room temperature.

One hundred microliters of blocking buffer (4X SSC containing 5% non-fat dry milk) was added to each slide, which was then covered with a coverslip and incubated for 20 minutes at room temperature. The coverslip was removed and 100 µl of avidin/fluorescein (5 µg/ml fluorescein avidin DCS (cell sorter grade, Burlingame, CA) in 4X SSC containing 0.05% Tween-20\) was added, the slide covered with a coverslip and allowed to incubate for 20 minutes at room temperature. The slide was then washed 3 times in 4X SSC containing 0.05% Tween-20 at room temperature for 3 minutes, followed by addition of 100 µl anti-avidin (5 µg/ml biotinylated, affinity-purified goat anti-avidin D (Vector) in 4X SSC containing 5% non-fat milk). The slide was covered with a coverslip and incubated at room temperature for 20 minutes. The slides were washed as above and a second fluorescein incubation was done using 100 µl avidin/fluorescein for 20 minutes at room temperature. In some cases the avidin/fluorescein steps were repeated one additional time. The slides were then washed two times in 4X SSC containing 0.05% Tween-20, at room temperature for 3 minutes, followed by one wash in 1X PBS at room temperature for 3 minutes.

The slides were then mounted in anti-fade medium (9 parts glycerol containing 2% 1,4-diazobicyclo-(2,2,2)-octane (DABCO) dissolved at 70°C, 1 part 0.2 M Tris/HCl, pH 7.5, and 0.25-0.5 µg/ml propidium iodide (Vector)).

The slides were viewed on an Olympus BH2-RFC microscope equipped with an Optronics ZVS-47E CCD RGB color video camera system (Goleta, CA). Images of the metaphase chromosome spreads were stored using Optimus software (Bothell, WA). Mapping of the GnRH-R II probe was carried out using the fractional length (FL) method (Z) on the previously ASG G-banded and photographed chromosome preparations. Digitized images from the same G-banded chromosomes were used in determining the corresponding FLqter values of the respective chromosome band boundaries with respect to the hybridized probe (Lichter et al., Science, 247:64-69, 1990).

Example 4
Localization of GNRHR-II in Brain

Animal and tissue preparation were as described in Quanbeck et al., J. Comp. Neurol. 380: 293-309, 1997. Briefly, the brain of one Rhesus Macaque monkey was immersed in a 4% paraformaldehyde in phosphate buffered saline (PBS) solution, pH 7.6 at embryonic day 70, for 1-8 hours. After fixation, the tissue was placed in 30% sucrose PBS solution, pH 7.6 until fully saturated. The tissue was then frozen and frontal sections of the brain were made with a cryostat at 12 μm. The slides were stored at -70°C until staining.

Polyclonal anti-peptide antibodies were prepared using standard techniques for antibody preparation. The peptide corresponded to 6 amino acids (Tyr Ser Pro Thr Met Leu Thr SEQ ID NO:7) of extracellular loop 3 of the human GnRH II and an N-terminal tyrosine for iodination, which was synthesized using an Applied Biosystems Model 431A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) according to manufacturer's instructions. This sequence was chosen because it differed markedly from the sequence in the type I GnRH receptor and this domain was shown to be responsible for ligand specificity (Flanagan

et al. J. Biol. Chem. 269:22636-41, 1994). The antibody was designated "ZGNRHRII-5".

Immunocytochemistry

To deactivate endogenous peroxidase, sections were washed four times at 15 minutes per wash with PBS, pH7.6, and treated with a 0.01% hydrogen peroxide in methanol solution. Sections were washed with PBS four times at 15 minutes per wash followed by blocking with 0.5% normal goat serum in PBS for two hours to remove non-specific binding. The slides were then exposed to either antiserum against human GnRH-R II extracellular loop 3 (ZGHRHII-5) at 1,000x concentration or GRF-6 (gift from N.M. Sherwood, Department of Biology, University of Victoria, Victoria, V8W 2Y2 Canada, a conjugate of salmon LHRH ([Trp^7-Leu^8] LHRH) to bovine thryoglobulin) at 6,000x concentration. The slides were stored in humidified chambers at 0°C to 4°C for 36 hours.

On the second day, sections were washed four times, 15 minutes per wash, with PBS and exposed to the second antibody (biotinylated goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA) for 1.5 hours at room temperature. This was followed by two, fifteen minute PBS washes, then exposure to avidin-biotin peroxidase complex solution (Elite, Vector Laboratory) for 1.5 hours. After two, fifteen minute washes with 0.05 M Tris-buffered saline solution, the final reaction product was visualized with a 3,3'-diaminobenzidine (DAB) solution (0.5% DAB with 0.01% hydrogen peroxide in 0.1 M Tris-buffered saline at pH7.6) that appears as a brownish-red grainy precipitate in the cell cytoplasm under light microscopy. Slides were cover-slipped in glycerol jelly. Data analysis was as described in Quanbeck et al., *ibid*. The distribution and type of neurons to which ZGHRHII-5 stained was similar to those stained with GF-6. The GnRH-R II receptor appears to co-localize with luteinizing hormone-releasing hormone (LHRH) in early LHRH neurons as described in Quanbeck et al. *ibid*.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ZymoGenetics, Inc.
1201 Eastlake Avenue East
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WA
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98102

and

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Cape Town
South Africa

(ii) TITLE OF INVENTION: Human Type II Gonadotropin-Releasing Hormone Receptor

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: ZymoGenetics, Inc.
(B) STREET: 1201 Eastlake Avenue East
(C) CITY: Seattle
(D) STATE: WA
(E) COUNTRY: USA
(F) ZIP: 98102

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lingenfelter, Susan E.

(B) REGISTRATION NUMBER: P41.156

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 206-442-6675
(B) TELEFAX: 206-442-6678

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1642 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGCATTTGGA AAATGTTAAA TTTTATTATT TAACATTCTT TACCATTATA GTTACTGCAC 60

ATAAGACTAT TACTACTAAA GGTCACTTCA GAGTCCCTGC AAAATGGCCT GGAATTTTGG 120

CAGCACCCAT TTTACACAAT ATTTCTTTTT CCACAAAATA ACAGACATAC CAGGAAAATC 180

ATTTCAGCTA AAAATATGAG TGAGGTGGTA GAAATATCAT CCCTTATAAA GCGCAATGTT 240

AGAATAGTAC TTGAGAAAGC AGGATTGTTT TAAGTTCCAA GATTTAACAA ACTTACTGTT 300

CAGCATCATA TTCAAGCCTA AAAGGAAGAT AGGATTTTCA AGATATATTT CCAACTTCTT 360

TAACATGGCA CCATGGATGA ACTGTTTCTC AGCACTGTGC TGCTTCACTT GGAATTAAGG 420

ATGAATTGGG AGGAGACAGT ATGACATAGG TGGGTATGTT GGGTGGTGAG GGGAACCAGT 480

TCTAATAGTC CTCAACTCCA CTCCAGCTGT TCCTGTTCCA CACGGTCCAC TGAGCTGGCC 540

CAGTCCCTTT CACTCAGTGT GTCACCAAAG GCAGCTTCAA GGCTCAATGG CAAGAGACCA 600

CCTATAACCT CTTCACCTTC TGCTGCCTCC TTCTGCTGCC ACTGACTGCC ATGGCCATCT 660

GCTATAGCCG CATTGTCCTC AGTGTGTCCA GGCCCCAGAC AAGGAAGGGG AGCCATGGTG 720

AGACTCCAAT TCCCAGGCCT TAATCCTTAA CCCTAGTCCT GTTGCCTCTA GCATCATTTA 780

TTTATCTACC TACCTAATAG CTATCTACCA GTCACTAAAC CATGGTGAGA TTCTAACCAT 840

GTCTAGCACC TGATGCTAGA GATAATTTTG TTGAATCCCT TCAATTATAA ACAGCTGAGT 900

TAGCTGGACA AGGACTAGGG AGGCAATCAG TATTATTTAT TCTTGAACAC CATCAAGTCT 960

AGACTTGGTG GCTTCATATT TCTATCATAA ACCCTGGGGG TAAGAAATCA TATAGTCCCA 1020

GGTTGGGAAG GGGAAAACGG TTTGCAACAT TCTCTCCTTG TAGGAGGCGA GCTCTGTCTC 1080

ACTAGCTATG CCCCTCCATC AATTCACCCT ATACTCAGAT CAGAAGCTGA GTGTCTGAAT 1140

TACAGTATAT TTTCTAAATT CCTAGCCCCT GCTGGTGAAT TTGCCCTCCC CCGCTCCTTT 1200

GACAATTGTC CCCGTGTTCG TCTCCGGGCC CTGAGACTGG CCCTGCTTAA CTTACTGACC 1260

TTCATCCTCT GCTGGACACC TTATTACCTA CTGGGTATGT GGTACTGGTT CTCCCCCACC 1320

ATGCTAACTG AAGTCCCTCC CAGCCTGAGC CACATCCTTT TCCTCTTGGG CCTCCTCAAT 1380

GCTCCTTTGG ATCCTCTCCT CTATGGGGCC TTCACCCTTG GCTGCCGAAG AGGGCACCAA 1440

GAACTTAGTA TAGACTCTTC TAAAGAAGGG TCTGGGAGAA TGCTCCAAGA GGAGATTCAT 1500

GCCTTTAGAC AGCTGGAAGT ACAAAAAACT GTGACATCAA GAAGGGCAGG AGAAACAAAA 1560

GGCATTTCTA TAACATCTAT CTGATCCTAA CAGAGTATGT AGGAACAGAA TAGTAAGTCT 1620

TTAGTGCCAT AAGATCTTAA CA 1642

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 600 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS

(B) LOCATION: 1..600

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 1               5                  10                  15

GCT CAA TGG CAA GAG ACC ACC TAT AAC CTC TTC ACC TTC TGC TGC CTC      96
Ala Gln Trp Gln Glu Thr Thr Tyr Asn Leu Phe Thr Phe Cys Cys Leu
            20                  25                  30

CTT CTG CTG CCA CTG ACT GCC ATG GCC ATC TGC TAT AGC CGC ATT GTC     144
Leu Leu Leu Pro Leu Thr Ala Met Ala Ile Cys Tyr Ser Arg Ile Val
        35                  40                  45

CTC AGT GTG TCC AGG CCC CAG ACA AGG AAG GGG AGC CAT GCC CCT GCT     192
Leu Ser Val Ser Arg Pro Gln Thr Arg Lys Gly Ser His Ala Pro Ala
    50                  55                  60

GGT GAA TTT GCC CTC CCC CGC TCC TTT GAC AAT TGT CCC CGT GTT CGT     240
Gly Glu Phe Ala Leu Pro Arg Ser Phe Asp Asn Cys Pro Arg Val Arg
65                  70                  75                  80

CTC CGG GCC CTG AGA CTG GCC CTG CTT AAC TTA CTG ACC TTC ATC CTC     288
Leu Arg Ala Leu Arg Leu Ala Leu Leu Asn Leu Leu Thr Phe Ile Leu
                85                  90                  95

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Cys Trp Thr Pro Tyr Tyr Leu Leu Gly Met Trp Tyr Trp Phe Ser Pro
            100                 105                 110

ACC ATG CTA ACT GAA GTC CCT CCC AGC CTG AGC CAC ATC CTT TTC CTC     384
Thr Met Leu Thr Glu Val Pro Pro Ser Leu Ser His Ile Leu Phe Leu
        115                 120                 125

TTG GGC CTC CTC AAT GCT CCT TTG GAT CCT CTC CTC TAT GGG GCC TTC     432
Leu Gly Leu Leu Asn Ala Pro Leu Asp Pro Leu Leu Tyr Gly Ala Phe
    130                 135                 140

ACC CTT GGC TGC CGA AGA GGG CAC CAA GAA CTT AGT ATA GAC TCT TCT     480
Thr Leu Gly Cys Arg Arg Gly His Gln Glu Leu Ser Ile Asp Ser Ser
145                 150                 155                 160
```

```
AAA GAA GGG TCT GGG AGA ATG CTC CAA GAG GAG ATT CAT GCC TTT AGA      528
Lys Glu Gly Ser Gly Arg Met Leu Gln Glu Glu Ile His Ala Phe Arg
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CAG CTG GAA GTA CAA AAA ACT GTG ACA TCA AGA AGG GCA GGA GAA ACA      576
Gln Leu Glu Val Gln Lys Thr Val Thr Ser Arg Arg Ala Gly Glu Thr
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AAA GGC ATT TCT ATA ACA TCT ATC                                      600
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 200 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Ala Gln Trp Gln Glu Thr Thr Tyr Asn Leu Phe Thr Phe Cys Cys Leu
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Leu Leu Leu Pro Leu Thr Ala Met Ala Ile Cys Tyr Ser Arg Ile Val
        35                  40                  45

Leu Ser Val Ser Arg Pro Gln Thr Arg Lys Gly Ser His Ala Pro Ala
    50                  55                  60

Gly Glu Phe Ala Leu Pro Arg Ser Phe Asp Asn Cys Pro Arg Val Arg
65                  70                  75                  80

Leu Arg Ala Leu Arg Leu Ala Leu Leu Asn Leu Leu Thr Phe Ile Leu
                85                  90                  95

Cys Trp Thr Pro Tyr Tyr Leu Leu Gly Met Trp Tyr Trp Phe Ser Pro
            100                 105                 110
```

Thr Met Leu Thr Glu Val Pro Pro Ser Leu Ser His Ile Leu Phe Leu
115 120 125

Leu Gly Leu Leu Asn Ala Pro Leu Asp Pro Leu Leu Tyr Gly Ala Phe
130 135 140

Thr Leu Gly Cys Arg Arg Gly His Gln Glu Leu Ser Ile Asp Ser Ser
145 150 155 160

Lys Glu Gly Ser Gly Arg Met Leu Gln Glu Glu Ile His Ala Phe Arg
165 170 175

Gln Leu Glu Val Gln Lys Thr Val Thr Ser Arg Arg Ala Gly Glu Thr
180 185 190

Lys Gly Ile Ser Ile Thr Ser Ile
195 200

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTGACCTTCA TCCTCTGCTG GACACC 26

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGAGAGCAGG AGTAGAAGTG AG 22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGTGTCCAGC AGAGGATGAA GGTCAG 26

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Tyr Ser Pro Thr Met Leu Thr
1 5

CLAIMS

We claim:

1. A polynucleotide which encodes a mammalian type II gonadotropin-releasing hormone receptor (GnRHR II) polypeptide.

2. The isolated polynucleotide of claim 1 wherein said polynucleotide encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 3.

3. An isolated polynucleotide of claim 2 wherein said polynucleotide comprises the sequence of SEQ ID NO: 2.

4. An isolated polynucleotide according to claim 1 wherein said polynucleotide is DNA.

5. An expression vector comprising the following operably linked elements:

a transcription promoter;

a DNA segment encoding a mammalian type II gonadotropin-releasing hormone receptor polypeptide; and

a transcription terminator.

6. An expression vector according to claim 5 wherein said polypeptide comprises the amino acid sequence of SEQ ID NO: 3.

7. A cultured eukaryotic cell into which has been introduced an expression vector according to claim 5, wherein said cell expresses a protein polypeptide encoded by the DNA segment.

8. A method for producing a mammalian type II gonadotropin-releasing hormone receptor polypeptide comprising:

culturing a cell into which has been introduced an expression vector according to claim 5, whereby said cell expresses a mammalian Type II gonadotropin-releasing hormone receptor polypeptide; and

recovering the mammalian Type II gonadotropin-releasing hormone receptor polypeptide.

9. An isolated polypeptide comprising a mammalian type II gonadotropin-releasing hormone receptor polypeptide.

10. An antibody that specifically binds to a polypeptide of claim 9.

11. A genomic DNA polynucleotide molecule comprising the sequence of SEQ. ID. NO. 1 wherein said genomic DNA polynucleotide molecule encodes a mammalian type II gonadotropin-releasing hormone receptor polypeptide.

12. A probe which comprises an oligonucleotide of at least 16 nucleotides, wherein the sequence of said oligonucleotide is at least 80% identical to the same-length portion of:

(a) SEQ ID NO:3; or

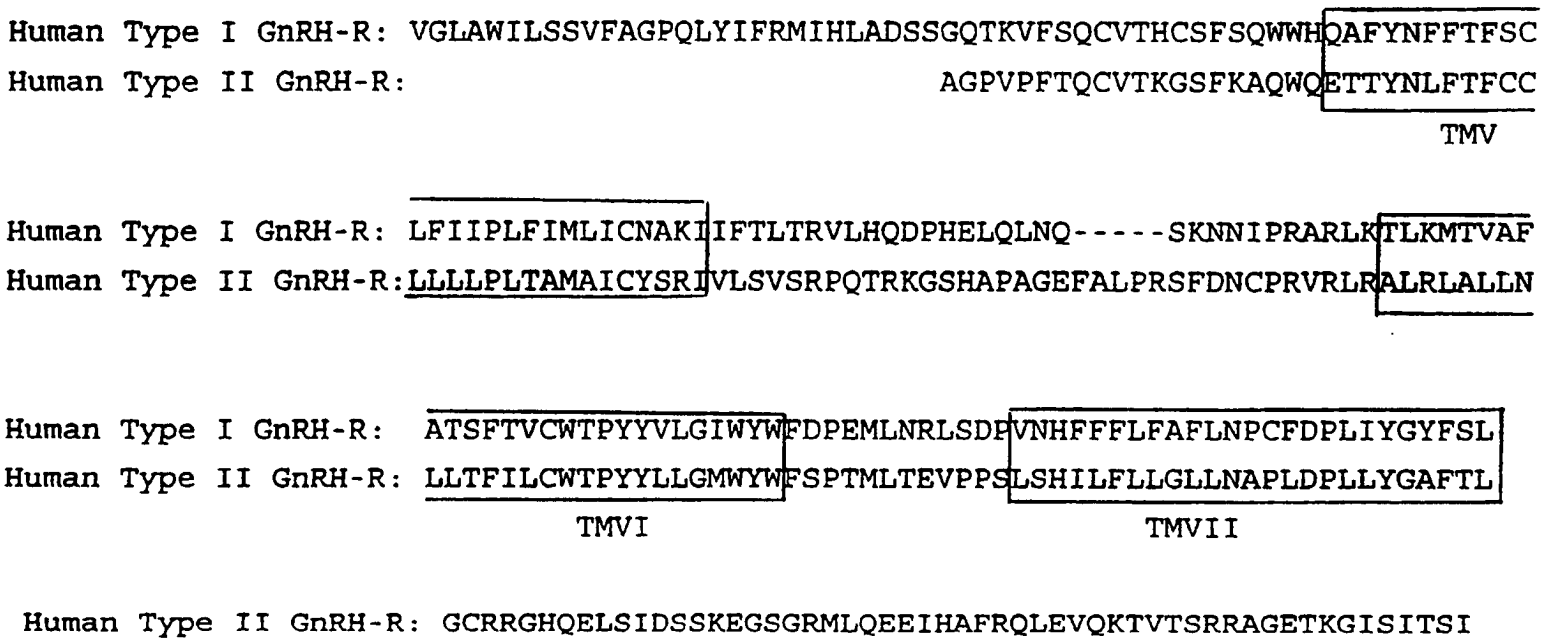
(b) a complement of a polynucleotide molecule that specifically hybridizes to (a).

13. A method for identifying a compound which modulates human type II gonadotropin-releasing hormone receptor-mediated metabolism in a cell, comprising:

incubating a test compound with eukaryotic cells which express recombinant type II human gonadotropin-releasing hormone receptor polypeptide on their surface; and

measuring the metabolism of the cells in the presence and in the absence of the test compound, or measuring the effect of a test compound on receptor (+) and receptor (-) cells,

wherein an increase in metabolism or effect above a control value indicates a test compound that modulates type II human gonadotropin-releasing hormone receptor mediated metabolism.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Human Type II GnRH-R: AGPVPFTQCVTKGSFKAQWQETTYNLFTFCC
TMV
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Human Type II GnRH-R:LLLLPLTAMAICYSRIVLSVSRPQTRKGSHAPAGEFALPRSFDNCPRVRLRALRLALLN
Human Type I GnRH-R: ATSFTVCWTPYYVLGIWYWFDPEMLNRLSDPVNHFFFLFAFLNPCFDPLIYGYFSL
Human Type II GnRH-R: LLTFILCWTPYYLLGMWYWFSPTMLTEVPPSLSHILFLLGLLNAPLDPLLYGAFTL
TMVI
TMVII
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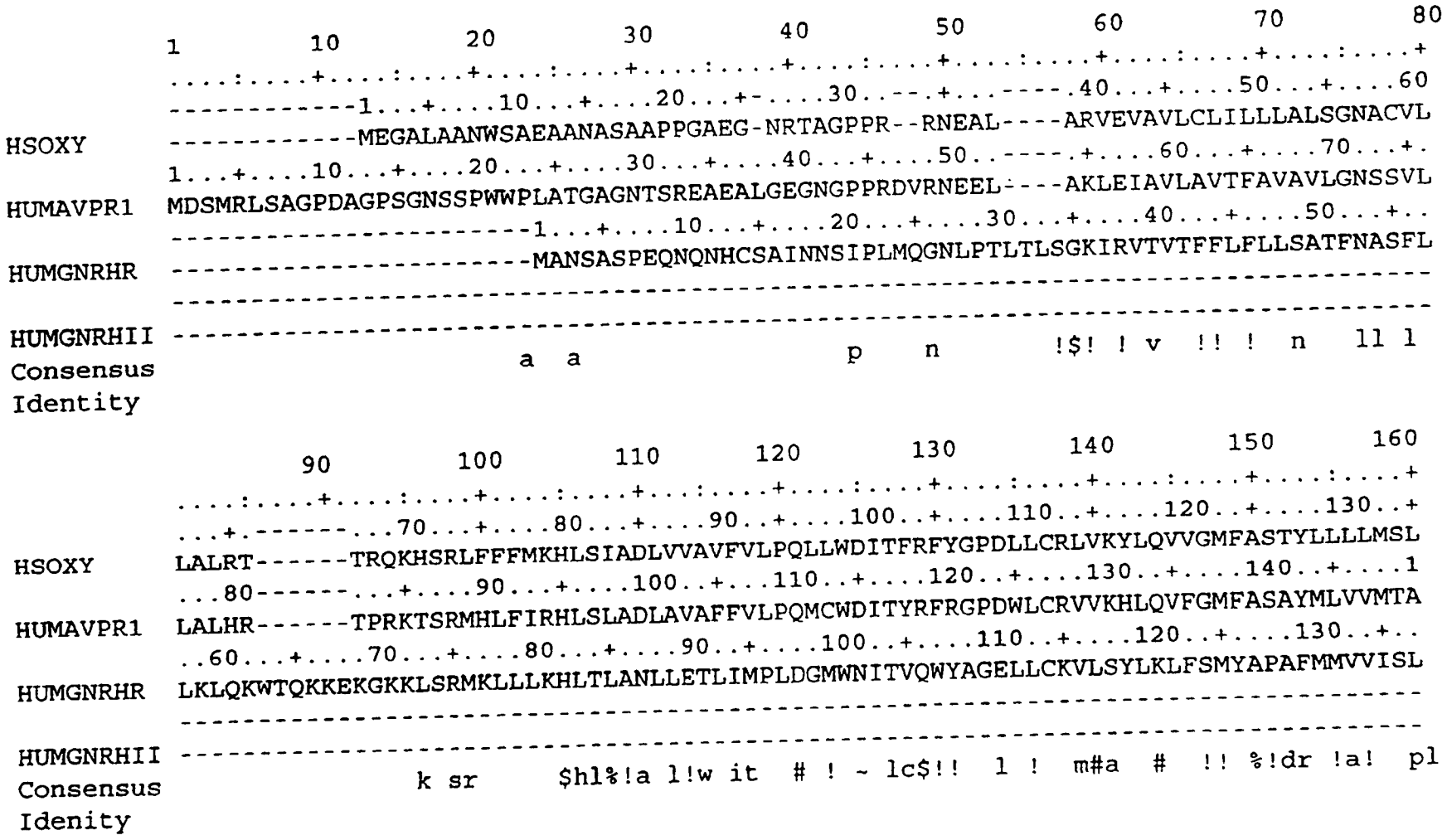
Figure 1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HUMAVPR1
HUMGNRHR
HUMGNRHII
Consensus
Identity
HSOXY
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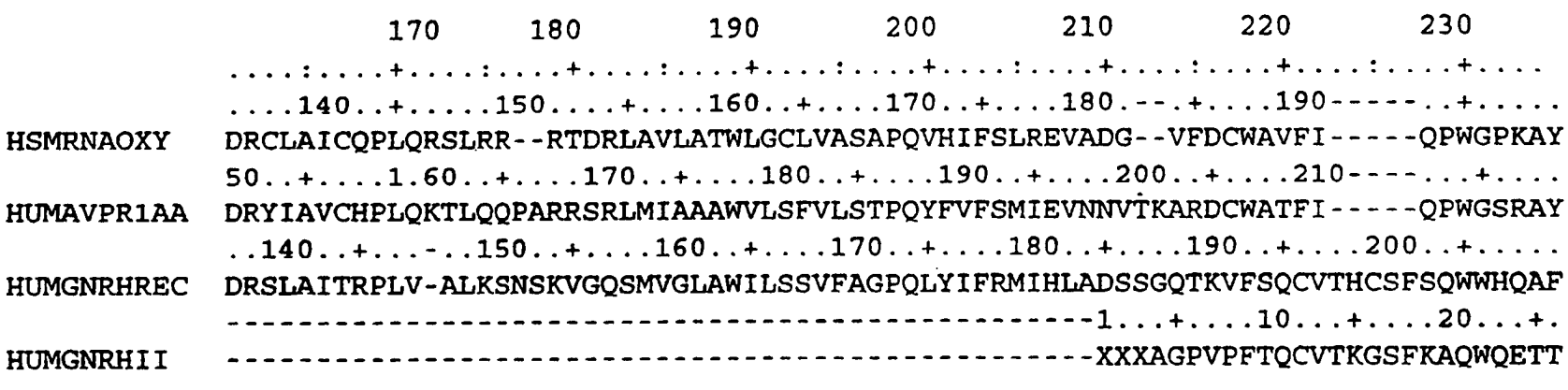
HUMGNRHR
HUMGNRHII
Consensus
Idenity

Figure 2A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HUMAVPR1AA DRYIAVCHPLQKTLQQPARRSRLMIAAAWVLSFVLSTPQYFVFSMIEVNNVTKARDCWATFI-----QPWGSRAY
HUMGNRHREC DRSLAITRPLV-ALKSNSKVGQSMVGLAWILSSVFAGPQLYIFRMIHLADSSGQTKVFSQCVTHCSFSQWWHQAF
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Consensus:
Identity:

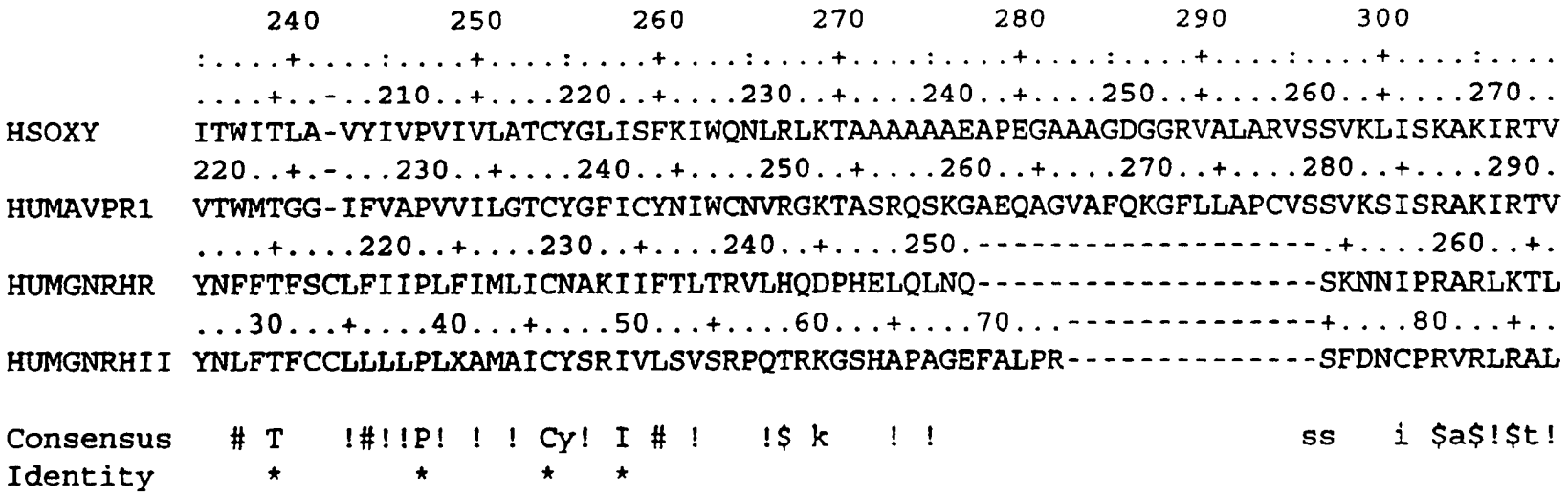
240 250 260 270 280 290 300
HSOXY ITWITLA-VYIVPVIVLATCYGLISFKIWQNLRLKTAAAAAAEAPEGAAAGDGGRVALARVSSVKLISKAKIRTV
HUMAVPR1 VTWMTGG-IFVAPVVILGTCYGFICYNIWCNVRGKTASRQSKGAEQAGVAFQKGFLLAPCVSSVKSISRAKIRTV
HUMGNRHR YNFFTFSCLFIIPLFIMLICNAKIIFTLTRVLHQDPHELQLNQ----------SKNNIPRARLKTL
HUMGNRHII YNLFTFCCLLLLPLXAMAICYSRIVLSVSRPQTRKGSHAPAGEFALPR----------SFDNCPRVRLRAL
Consensus
Identity

Figure 2B

```
           310       320       330       340       350       360       370       380
           +....:....+....:....+....:....+....:....+....:....+....:....+....:....+....:
           +....280..+....290..+....300----..+....310..+....320..+....330..+....340..+..
HSOXY      KMTFIIVLAFIVCWTPFFFVQMWSVWDA---NAPKEASAFIIVMLLASLNSCCNPWIYMLFTGHLFHELVQRFLCC
           .+....300..+....310..+....320..+....330..+....340..+....350..+....360..+....
HUMAVPR1   KMTFVIVTAYIVCWAPFFIIQMWSVWDPMSVWTESENPTITITALLGSLNSCCNPWIYMFFSGHLLQDCVQSFPCC
           ...270..+....280..+....290..+....300..+....310..+....320..+...
HUMGNRHR   KMTVAFATSFTVCWTPYYVLGIWYWFDPEMLNRLSDPVNHFFFLFAFLNPCFDPLIYGYFSL
           ..90...+....100..+....110..+....120..+....130..+....140..+....150..+....160.
HUMGNRHII  RLALLNLLTFILCWTPYYLLGMWYWFSPTMLTEVPPSLSHILFLLGLLNAPLDPLLYGAFTLGCRRGHQELSIDSS

Consensus  kmt !!!t #!vcw p##!!  w  #d        ~     ! !!l! !      #!      $     !
Identity

              390       400       410       420       430       440
           ....+....:....+....:....+....:....+....:....+....:....+
           ..350..+....360..+-------....370-------....380..+...
HSOXY      SASYLKGRRLGETSASKK----SNSSS-------FVLSHRSSSQRSCSQPSTA
           370..+....380..+....390..+....400..+....410..+....420
HUMAVPR1   QNMKEKFNKEDTDSMSRRQTFYSNNRSPTNSTGMWKDSPKSSKSIKFIPVST
           .+....170..+....180..+....190..+....200.
HUMGNRHII  KEGSGRMLQEEIHAFRQLEVQKTVTSRRAGETKGISITSI
Consensus                                          SS        ST
Identity                                           **        **
```

Figure 2C

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/10144

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/72 C12N15/85 C12N5/10 C07K16/28
C12Q1/68 G01N33/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	WO 94 00590 A (THE MT. SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK) 6 January 1994 see page 6, line 10 - page 43, line 13 ---	1,4,5, 7-10,13 2,3,6, 11,12
X A	EP 0 678 577 A (TAKEDA CHEMICAL INDUSTRIES, LTD.) 25 October 1995 see page 3, line 10 - page 6, line 35 see page 7, line 3 - page 11, line 24 --- -/--	1,4,5, 7-10,13 2,3,6, 11,12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 September 1997	06.10.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)